THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 15, Issue of August 10, pp. 5424-5427, 1973
Printed in U.S.A.

Protein Hydration Changes in the Formation of the Nicotinamide Adenine Dinucleotide Complexes of Glyceraldehyde 3-Phosphate Dehydrogenase of Yeast

II. THE SPIN LATTICE RELAXATION OF SOLVENT WATER PROTONS*

(Received for publication, March 1, 1973)

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SUMMARY

The glyceraldehyde 3-phosphate dehydrogenase of yeast is known to undergo a particle volume contraction and the commensurate loss of a hydration component when it forms a complex with NAD. We have inquired whether the water movement in the conformational transition can be monitored by measurements of the spin lattice relaxation rate, T_1^{-1} of solvent water protons. In solutions of diamagnetic proteins, a class of bound water molecules is rapidly relaxed in the protein hydration domain and the relaxation is averaged in the bulk solvent by water exchange. T_1^{-1} was found by pulse NMR (nuclear magnetic resonance) measurements to increase as a linear function of the apoprotein concentration. From measurements at two frequencies, the correlation time was calculated to be in the range expected from the rotational diffusion relaxation time of the protein. The minimal number of exchangeable bound water molecules required to account for the apoprotein effect is 26 per protein subunit, a small fraction of the total hydration of the protein. NAD complex formation produces a further increase in the relaxation rate of the solvent water protons. Increments of T_1^{-1} as a function of NAD concentration follow the course of a binding isotherm with a stoichiometry of 1 mole of NAD per mole of protein subunit. The direction of the NAD effect corresponds to an increase in the class of bound water molecules that contribute to the relaxation rate increase. Hence, different classes of bound water are represented by the T_1^{-1} effect and by the larger amount of water eliminated in the conformational transition. The latter water is loosely bound or trapped in cavities of the protein structure, and differences in its proton relaxation rate, in and out of the hydration domain, are not detected by NMR in the frequency range examined. The number of specifically bound water molecules involved in the T_1^{-1} effect of NAD is estimated to be small enough to be accounted for by local effects at the binding sites.

Enhancements of the spin lattice relaxation rates of solvent water protons have been extensively utilized to monitor the interactions of paramagnetic ions with enzymes and their substrates (1). To a lesser extent, the water proton relaxation rate is also increased by diamagnetic proteins in the absence of paramagnetic ions. An analysis of the latter effect has indicated that the enhancement may be produced by a relatively small number of water molecules in the protein hydration domain that are irrotationally bound, or nearly so. These molecules are rapidly relaxed by viture of a greatly increased correlation time and are exchanged with the bulk solvent (2). The effective water-binding sites have not been further defined. The present report describes a limited approach to the problem by examination of the effects on water proton relaxation rates of a ligand addition that perturbs the solvation domain of a protein. It has been observed that the glyceraldehyde 3-phosphate dehydrogenase of yeast undergoes a substantial volume contraction (3) and a nearly equivalent decrease in preferential hydration (4) when the NAD complex is formed. Since it was found that the structural perturbation involves the extrusion of water from cavities of the protein structure that exclude the entrance of phosphate ions, it seemed possible that the motion of such water molecules, if exchangeable, might be sufficiently restricted to allow them to contribute to the relaxation enhancement by the apoprotein. NAD complex formation would then partially reverse the enhancement. To test this hypothesis, solvent water proton relaxation rates over a temperature and pH range have been measured by

* This work was supported by Grant AM-13115 from the National Institutes of Health and by Grant GH-31071 from the National Science Foundation.

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pulse nuclear magnetic resonance at fixed protein concentrations as a function of the concentration of NAD.

MATERIALS AND METHODS

The glyceraldehyde 3-phosphate dehydrogenase was prepared from Fleishmann's bakers' yeast as previously described (4). It was prepared at 1-month intervals during which time it exhibited specific activities in the direction of NADH oxidation of 140 to 180 i.u. mg^{-1}. The pyridine nucleotides were products of Sigma Chemical Co.

Proton relaxation measurements in solutions of enzyme and enzyme-NAD were made by the 180-90 pulse sequence method (5) at 30 and 3.5 MHz in a laboratory-built pulse NMR spectrometer (6). Sedimented crystal cakes of the apoenzyme from concentrated ammonium sulfate solutions were dissolved in the desired buffer and equilibrated with the buffer by dialysis or by passage through a column (40 cm) of Sephadex G-25. The test samples (2.0-ml volume) were placed in tubes the diameters of which were approximately equal to the sample height. Relaxation times were obtained from curves consisting of seven time points repeated four to eight times, and the results were fed on line to a PDP-11 computer for signal averaging, statistical evaluation, and computation of the time constants. Temperature control of the pre-equilibrated solutions was maintained by a thermostated air stream. The time intervals between pairs of pulses, controlled by return to M_0, were $15 \times T_1$. New aliquots of enzyme, 60 mg each, were used for each NAD concentration tested. Four different enzyme preparations were employed.

RESULTS AND DISCUSSION

The relaxation rate enhancement of water protons by proteins is defined as the difference in the relaxation rate, T_1^{-1}, of water protons in the protein solution and in the solvent in the absence of protein and is described by Equation 1 (2)

$$\left(T_1^{-1}\right)_{soln} - f_w\left(T_{1w}^{-1}\right) = f_b\left(T_{1b} - \tau_m\right)^{-1} \qquad (1)$$

The T_1 values are relaxation times, the subscripts w and b refer, respectively, to free and bound water, and f_w and f_b are the effective mole fractions of water in the two environments. Water protons are rapidly relaxed on the protein, where they stay with the residence time τ_m and are exchanged with the bulk solvent. The T_1 values are described by a well established relation of the form of Equation 2 (7)

$$T_1^{-1} = K\left[\frac{\tau_c}{1+(\omega\tau_c)^2} + \frac{4\tau_c}{1+4(\omega\tau_c)^2}\right] \qquad (2)$$

where K is an assembly of constants, ω is angular frequency, and τ_c is the correlation time characteristic of the molecular motion. If the effective exchangeable water, designated by f_b, is rigidly bound, its correlation time is $\tau_c = \tau_D/3$, where τ_D is the rotational diffusion relaxation time of the protein (8). In order to detect the protein effect, it is necessary in such a case to work at frequencies in the range of, or less than, τ_c^{-1}. In our probes with dehydrogenase at 30 MHz, the enhancement by protein was small and NAD effects were not detected. However, at 3.5 MHz, our lowest available frequency, the enhancement by apoprotein and its modulation by NAD were measurable with an acceptable signal to noise ratio. The enhancement by apoprotein increased as a linear function of protein concentration in the range of 10 to 60 mg ml^{-1}, and a standard protein concentration of 30 mg ml^{-1}, or 0.208 mM, was selected for titration.

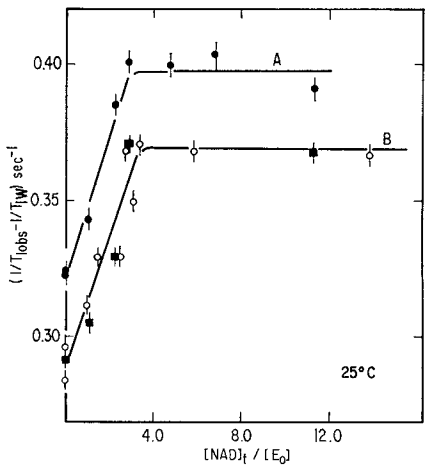

FIG. 1. Solvent water proton relaxation rate enhancements in the titration of the glyceraldehyde 3-phosphate dehydrogenase of yeast with NAD (at 25°). *A*, 0.05 M sodium pyrophosphate, 2 mM EDTA, pH 8.5; *B*, 0.05 M potassium phosphate, 0.05 M potassium chloride, 2 mM EDTA; ■——■, pH 6.0; ○——○, pH 7.4.

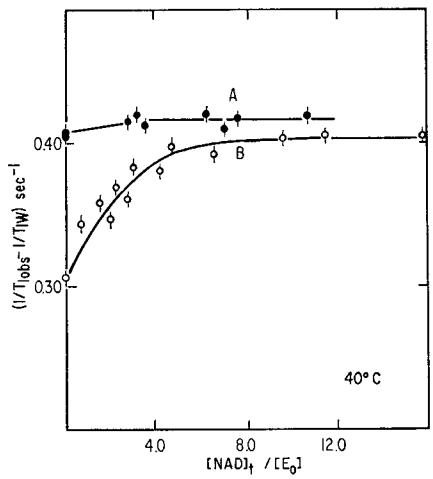

FIG. 2. Solvent water proton relaxation rate enhancement in the titration of the glyceraldehyde 3-phosphate dehydrogenase of yeast with NAD at 40°. *A*, pH 8.5 in 0.05 M sodium pyrophosphate and 2 mM EDTA; *B*, pH 7.4 in 0.05 M potassium phosphate, 0.05 M potassium chloride, 2 mM EDTA.

Titration Curves—The titrations of apoprotein with NAD, monitored by T_1 measurements at pH 6.0 to 8.5 and at 25°, are shown in Fig. 1. The enhancements increase with NAD concentration and level off at or near the expected stoichiometry of 4 moles of NAD per mole of tetrameric protein. Because of the high protein concentrations relative to the dissociation constants of the complexes (4, 9) the binding curves are essentially linear. The magnitude of the enhancement is pH-independent. Results with NAD at a higher temperature are shown in Fig. 2. The NAD effect at pH 7.4 and 40° is slightly smaller than at 25°, and there is a sign of some dissociation as saturation is approached. However, at pH 8.5 and 40°, the enhancement by apoprotein is exceptionally high, and there is only a marginal addition enhancement by the ligand. The latter results were obtained with two independent enzyme preparations and were not due to contamination or enzyme inactivation. Table I summarizes the numerical results.

Correlation Times—The value of τ_c for the water that con-

tributes to the T_1^{-1} increase by the apoprotein can be obtained from the ratio of the enhancements at 3.5 and 30 MHz (Equation 3)

$$\frac{(T_1^{-1})_{3.5}}{(T_1^{-1})_{30}} = \frac{\left[\frac{1}{1+(\omega_{3.5}\tau_c)^2} + \frac{4}{1+4(\omega_{3.5}\tau_c)^2}\right]}{\left[\frac{1}{1+(\omega_{30}\tau_c)^2} + \frac{4}{1+4(\omega_{30}\tau_c)^2}\right]} \quad (3)$$

From Table I the experimental ratio is 0.285/0.067 = 4.254. To solve for τ_c, it was convenient to use Equation 3 in the following form

$$\frac{(T_1^{-1})_{3.5}}{(T_1^{-1})_{30}} = \frac{(\omega_{30}\tau_c)^2}{2}\left[\frac{1}{1+(\omega_{3.5}\tau_c)^2} + \frac{4}{1+4(\omega_{3.5}\tau_c)^2}\right] f(\tau_c) \quad (4)$$

where $f(\tau_c)$ is given by

$$f(\tau_c) = \frac{1 + \frac{5}{4(\omega_{30}\tau_c)^2} + \frac{1}{4(\omega_{30}\tau_c)^2}}{1 + \frac{5}{8(\omega_{30}\tau_c)^2}} \quad (5)$$

In the limit

$$(\omega_{30}\tau_c)^2 \gg 1, \quad f(\tau_c) \equiv 1$$

We employed an alternative procedure in which $f(\tau_c)$ was initially chosen to be unity, and τ_c was then determined from the remaining quadratic equation. This value for τ_c was then used to calculate a better value for $f(\tau_c)$ and the calculation was repeated. The results converged to a final value, $\tau_c = 0.60 \times 10^{-8}$ s.

Since the dehydrogenase is a relatively compact globular protein with an apparent axial ratio in solution of about 0.6 (3), it may be treated, to a first approximation, as a sphere with a rotational diffusion relaxation time (Equation 6)

$$\tau_D = \frac{4\pi\eta r^3}{kT} \quad (6)$$

where r is the molecular radius and η is the viscosity of the solvent. Hence

$$\tau_c = \tau_D/3 = V\eta/kT$$

where V is the molecule volume. For a molecular weight of 144,000 and an anhydrous partial specific volume of 0.74, $V = 17 \times 10^{-20}$ cm^3 and $\tau_c = 3.8 \times 10^{-8}$ s. If we use the expanded particle volume of 26×10^{-20} cm^3 obtained at pH 8.5 and 40° by x-ray scattering (3), $\tau_c = 5.7 \times 10^{-8}$ s. Thus, within an order of magnitude, the result obtained from independent measurements of molecular parameters and viscosity agrees with the result from NMR. However, the difference may be real and suggests that the effective exchangeable water is not irrotationally bound but has some independent degrees of freedom.

Effective Water-binding Sites—An estimate of the minimum number of exchangeable water molecules required to produce the observed proton relaxation increase by the apoprotein may be obtained from Equation 1. The correlation time, τ_{cw}, of free water is about 3×10^{-12} s and its observed relaxation time at 25° is 2.76 s. Taking the NMR value for the correlation time of the effective bound water, $\tau_c = 0.60 \times 10^{-8}$ s, T_{1b} may be calculated from the relation $T_{1b}/T_{1w} = \tau_{cw}/\tau_{cb}$ and is found to be 14×10^{-4} s. The mole fraction of free water, f_w, may be taken as unity. If τ_m is small compared with T_{1b}, then Equation 1 may be solved for f_b, which is found to be 3.9×10^{-4}. In 55 M water the effective bound water concentration is therefore 21 mM, or 21/0.208 = 103 water molecules per molecule of protein. A number in this range per unit weight of protein has been obtained by Koenig and Schillinger (2) in a similar calculation from NMR data for apotransferrin. These authors have also summarized evidence against major contributions to relaxation rate increase from dissociable protons of the protein in the pH range of interest. The absence of an NAD effect on T_1 at pH 8.5 and 40° may involve ionization effects under those conditions, but it requires further investigation.

NAD Effect—The additional relaxation rate increase produced by saturation of the binding sites of the protein with NAD is about 25% of that produced by apoprotein alone. If this involves a proportional increase in the f_b hydration component discussed above, the number of water molecules transferred to the f_b hydration class from within or outside the hydration domain is about 6.5 molecules of water per molecule of NAD bound. This increase is in contrast with the protein conformational effect of NAD binding which results in the release from an internal hydration component of at least 120 molecules of water per molecule of bound NAD (4). According to these calculations, the number of water molecules involved in the T_1 effect of NAD is sufficiently small to be accounted for by a local effect at the binding site, independent of the NAD-induced protein conformational transition.

We infer that the water molecules released by the protein contraction have too rapid a Brownian motion in the bound or trapped form to contribute to the T_1^{-1} enhancement by the apoprotein. A large number of water molecules specifically bound to a protein have recently been resolved by neutron diffraction analysis of myoglobin crystals (10). It is likely that a significant number of such molecules, hydrogen-bonded, for example, to carbonyl groups on the dehydrogenase surface, would remain sufficiently immobilized in solution and would exchange at a rate that would account for the observed relaxation rate increase.

TABLE I

Increases in solvent water proton relaxation rates in solutions of glyceraldehyde 3-phosphate dehydrogenase of yeast and its saturated NAD complex

Frequency	Temperature	pH	$(T_1^{-1})_{obs} - (T_1^{-1})_w$	
			Apoenzyme	$E(NAD)_4$
MHz			s^{-1}	
3.5	40°	7.4	0.309	0.400
	25	7.4	0.283	0.387
	25	6.0	0.292	0.398
	25	8.5	0.330	0.399
	40	8.5	0.411	0.415
30.0	25		0.067	0.066

Acknowledgments—We would like to thank Mr. Pei-Pin Ho for assistance in making some of the measurements and Dr.

Mildred Cohn for the opportunity afforded one of us (S. F. V.) to make an exploratory probe of the T_1 effects in her laboratory at the Johnson Foundation.

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